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ORIGINAL ARTICLE

Micropropagation via somatic embryogenesis of *Iris pallida* Lam. ecotypes

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Abstract

Iris pallida LAM., which has characterized the economy of small and medium-sized Tuscan farms for decades, is one of the most typical rustic species of the Mediterranean landscape. *I. pallida* essence is widely used in the perfumery sector for the indicative smell of violet caused by its high content of irones, ketone compounds accumulated inside the rhizome during their storage phase. One of the main critical aspects of its cultivation is the vegetative propagation method, traditionally carried out by rhizome transplanting, which does not allow obtaining a sufficient number of plants to encourage its cultivation. The state of the art indicates the micropropagation of *I. pallida* via somatic embryogenesis as the most effective propagation method, using immature flower components as starting tissue; however, there are no studies aimed at comparing the responses of different ecotypes of *I. pallida* to this technique. In this study, *I. pallida* clones of HE, VIC and BA ecotypes were obtained via somatic embryogenesis, starting from bud (young and immature) and leaf explants, monitoring all the development steps from callus to plantlets production, and testing clones adaptation to field conditions, using a modified acclimatization protocol. The results highlighted the different responses of the ecotypes to the in vitro protocol and demonstrated the effectiveness of somatic embryogenesis in producing *I. pallida* plantlets with a chromatographic profile overlapping with the donor plant; this alternative propagation method could allow to produce plantlets without sacrificing sealable rhizome.

Key message

I. pallida ecotypes respond differently to somatic embryogenesis; the technique showed to be valuable for new plants obtaining, opening a new potential way to *I. pallida* industrial in vitro production.

Keywords Orris · In vitro · Buds · Leaves

Introduction

The essence of *Iris pallida* Lam. is one of the most appreciated fragrances in perfumes industry, thanks to its characteristic powdery and woody scent. Originally from central Europe, this species is traditionally cultivated in the Italian areas of Pratomagno and Chianti Fiorentino (Belletti et al. 2013). Its rhizomes are sold to industries, specialized distillation of raw materials and essential oils extraction, to produce orris violet-like essence (de Bonneval et al. 2020). The aromatic characteristics of its essence are determined by ketone compounds called irones, generated by the oxidation of iridals, triterpenoids which accumulate in the rhizome during the years of cultivation (Krick et al. 1983; Bicchi et al. 1993). The interest in *I. pallida* cultivation is increasing among perfume industries, mostly based in France and

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Germany; however, in recent decades, orris production in Italy significantly decreased, because of the competitive prices at which rhizomes produced in Morocco and China were sold (Ieri et al. 2022). *I. pallida* cultivation requires at least three years in the field. Then, rhizomes are harvested, cut into slices and left to dry in ventilated rooms for other three years. After being harvested, a substantial part of rhizomes collected needs to be transplanted in field to restart orris cultivation, causing a significant loss of income to the farmer. This critical aspect needs to be investigated, to find an alternative type of propagation that can produce *I. pallida* plants without using saleable material. Previous studies focused on the in vitro micropropagation, widely used not only to obtain a vegetative propagation of ornamental and medicinal species, but also to preserve wild plants and species in danger of extinction (Safari et al. 2023; Lambardi et al. 2013). Being orris propagation by seeds difficult to perform because of their low germination ability (Simonet 1932), the in vitro micropropagation was mainly done through shoots and bulbils proliferation of *Iris* species interesting from an ornamental point of view (Ascough et al. 2009). Another commonly used technique for *Iris spp.* micropropagation is the somatic embryogenesis, performed starting from reproductive explants (Tikhomirova 2020; Safari et al. 2023). The effectiveness of this technique is strongly linked to the youth of the explant and the ecotype from which the explant is obtained (Vidal et al. 2009). Only three studies performed somatic embryogenesis technique on *I. pallida* starting from leaves and flower buds, setting up a disinfection protocol specifical for the explants used or testing different types of substrates, for callus proliferation and plantlets obtaining (Lucchesini et al. 2017; Jéhan et al. 1994; Gozu et al. 1993); however, none of these studies have developed a reproducible and complete protocol ready to be used by nurseries to propagate new *I. pallida* varieties with commercial value In this study, somatic embryogenesis was performed on clones obtained from different *I. pallida* ecotypes, modifying the pre-existing protocol identifying the critical aspects of the procedure (maturity of the starting explant, media composition, pre-acclimatization phase). Testing this technique on different ecotypes of *I. pallida* traditionally cultivated in Italy and France for essential oil production, could identify those more suitable for in vitro industrial production by companies specialized in the micropropagation sector. This study could therefore contribute to solve the critical phase of *I. pallida* propagation, reducing farmers economic losses and incentivizing its cultivation.

Materials and methods

Somatic embryogenesis on young flower buds

Young flower buds were taken from *I. pallida* plants belonging to three different ecotypes, namely GR, HE and SP, previously transplanted in the experimental fields of the Department of Agricultural, Food and Agri-environmental Sciences of the University of Pisa (DiSAAA-a). The side buds were taken in April from completely developed flower stems, and their outer sheath was removed; buds were then rinsed with tap water for 20 min and subjected to a disinfection protocol by submerging buds for 30 s in 70% ethanol (EtOH), and then in a solution made of 15% sodium hypochlorite (NaClO). Finally, explants consisting of the inner tepals, the youngest and less contaminated part of the bud, were obtained under laminar flow hood, transferred to Petri® dishes containing a callus induction medium (CI, Table 1) and placed in a growth chamber in the dark

Table 1 Composition of culture media used for somatic embryogenesis of *I. Pallida* ecotypes

Media composition	CI callus induction	EI embryos induction	G germination	M multiplication
Macroelements	MS	MS	MS	MS
Microelements	MS	MS	MS	MS
Vitamins	MS	MS	Gamborg B5	Gamborg B5
Sucrose	50 g L^{-1}	50 g L^{-1}	20 g L^{-1}	30 g L^{-1}
MES	500 mg L^{-1}	500 mg L^{-1}	500 mg L^{-1}	500 mg L^{-1}
GSH	300 mg L^{-1}	300 mg L^{-1}	300 mg L^{-1}	300 mg L^{-1}
Hydrolysed caseine	250 mg L^{-1}	250 mg L^{-1}		
Proline	250 mg L^{-1}	250 mg L^{-1}		
Tyrosine	250 mg L^{-1}	250 mg L^{-1}		
2.4-D	1 mg L^{-1}	1 mg L^{-1}		
Kinetin	1 mg L^{-1}	0.1 mg L^{-1}		
BA			0.1 mg L^{-1}	0.1 mg L^{-1}
NAA			0.1 mg L^{-1}	0.1 mg L^{-1}
Gelrite	3 g L^{-1}	3 g L^{-1}	3 g L^{-1}	3 g L^{-1}
pH	5.9	5.9	5.9	5.9

at 22 °C, changing the medium every month. After four months, calli obtained were transferred to an embryo induction medium, called EI (Table 1). After two weeks, embryos formed were moved to a germination medium called G (Table 1) to stimulate embryo conversion into plantlets. During the experiment, the percentage of callus formed on CI was monitored, and callus growth was measured weekly before being transferred to EI medium, starting from part of fresh callus on average 200 mg. Then, embryos regenerated (n° of embryos g_{fw} $callus^{-1}$) was monitored and plantlets obtained (n° plantlets from a single initial explant) were investigated. Plantlets obtained were subsequently transferred to Magenta® vessels containing 50 ml of M medium (Table 1). Every three weeks, new sprouts were obtained, separated and transferred to new Magenta® vessels (3/5 shoots for each vessel), taking at each subculture the number of sprouts produced from each initial explant to obtain the multiplication rate of each ecotype.

Somatic embryogenesis on clone HE leaf tissue

The somatic embryogenesis technique was also tested using leaf tissue taken from micropropagated shoots of clone HE. Leaves basal portions were selected, cut into two different dimensions (5 and 3 mm) and established on a callus induction medium (CI) to find out the best one in terms of callus formed. The steps were done using the same procedure performed with young flower buds. During the experiment, the percentage of callus formed was monitored and calli obtained were weighted, starting from pieces of fresh callus on average 200 mg, to investigate their monthly weight gain before being transferred to EI medium. Then, the number of embryos and regenerated plantlets were investigated as for young flower buds.

Somatic embryogenesis on immature flower buds of clone BA and clone VIC

The somatic embryogenesis technique using young flower buds performed during the first experiment appeared to be successful. Therefore, we decided to improve this technique on the ecotypes that showed a better response during the first trial (clone BA and clone VIC), taking flower buds at the beginning of floral stem development. Buds were deprived of their outer sheath and explants consisting of both outer and inner tepals were excised, disinfected with the above procedure and transferred on CI medium. All the following steps were done as described above for young flower buds and leaves.

Acclimatization of micropropagated plantlets of clone BA and clone VIC

Thirty in vitro plantlets for each ecotype were transferred to Magenta® vessels with ventilated cap for the pre-acclimatization phase; after a month, plantlets were transferred to plastic jars with autoclaved perlite and placed in a growth chamber with sodium lamps (400 W), at 26 °C under 16-hour photoperiod (400 µmol $m^{-2}s^{-1}$), covered with a plastic film and sprayed with a nutrient solution containing Murashige & Skoog macro and micro salts (1962) and Gamborg (1968) B5 vitamins. Thereafter, they were gradually acclimatized to the growth chamber environmental conditions by shedding the plastic film every day for an increasing number of hours until it was completely removed. After two weeks, plants were transferred to a mix of soil and perlite in a 1:1 ratio. They were then adapted for a week to environmental conditions keeping them outside, and finally transplanted to the field. At the end of the acclimatization process the percentage of survival was monitored.

Iridals phytochemical profile of clone VIC

Four replicates, each consisting of the rhizome of an individual plant belonging to VIC clone, were analysed to compare the phytochemical profile of *I. pallida* mother plants and micropropagated plants after acclimatization. The rhizomes were cleaned, peeled, cut into small pieces and stored at -80 °C, until acetone extraction was performed (Gozu et al. 1993). The rhizome tissue (1 g for each replicate) was chopped in a mortar with 5 mL acetone, transferred into a 10 mL test tube in an ice bath, kept under sonication for one hour and stored overnight at -20 °C. Then the supernatant was collected, and the pellet was extracted again with 5 mL fresh acetone and re-sonicated for an hour, storing it again overnight at -20 °C. For each extract, the two supernatant aliquots were pooled and filtered with Chromafil® 0.20 µm PTFE membrane, 25 mm diameter syringe filters (Macherey-Nagel, Düren, Germany). Four leaf explants (100 mg for each replicate) were taken from four *I. pallida in vitro* plantlets clone VIC, and extracted following the same procedure above described. The HPLC separations were carried out with a PU-2089 quaternary pump and a four-channel UV-2077 detector (Jasco, Tokyo, Japan), using a C18 column (Nucleodur®, 250/4.6, 100-5; Macherey-Nagel, Düren, Germany). The analyses were performed at room temperature with 20 µL injection volume and 1 mL min^{-1} flow rate, using methanol (solvent A) and water (solvent B) with the following elution gradient: 0–2 min, A 75%; 2–22 min, A 75–100%; 22–28 min, A 100%; 28–28.5 min, A 100 – 75%; 28.5–30 min, A 75%. The working wavelengths were 265, 254, 245 and 235 nm. For the identification of the iridals

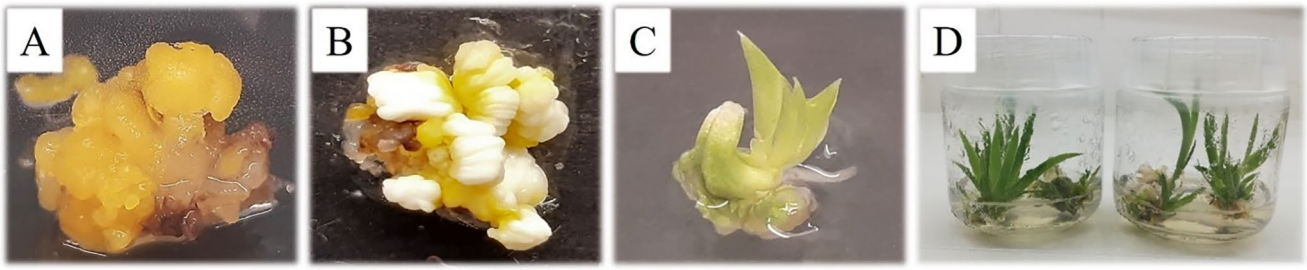

Fig. 1 Steps of somatic embryogenesis technique performed on *I. pallida* clone HE buds: (**A**) Callus formation; (**B**) Embryos germination; (**C**) Embryos conversion into new plantlets; (**D**) Micropropagated plantlets

analyses were carried out by Sciex 5500 QTrap + mass spectrometer (AB Sciex LLC, Framingham, MA, USA), using a Phenomenex Kinetex® 2.6 µm Biphenyl 100 Å LC column 100 × 2.1 mm (Phenomenex, Torrance, CA, USA) to perform chromatographic separation. Since the pure standard compounds are not commercially available for the iridals, both iripallidal and iriflorental were quantified as α-ionone (Sigma Aldrich, Milan, Italy).

Statistical analysis

All data were expressed as the mean ± S.E. of the number of replicates; percentage data underwent to angular transformation before proceeding to t-test and analysis of variance (ANOVA). A student t-test or analysis ANOVA was performed at a $p \leq 0.05$ significant level. The mean values were separated by the Tukey post-test ($p < 0.05$). Statistical analysis was carried out with GraphPad Prism (version 10.00 for Windows, GraphPad Software, La Jolla, San Diego, CA, USA).

Results

Somatic embryogenesis on young flower buds

In this first experiment, the modified Lucchesini et al. (2017) regeneration protocol by somatic embryogenesis was applied on three *I. pallida* ecotypes traditionally cultivated in Italy and France for production of essential oils. During the first steps of callus formation on CI medium, clone HE responded better in terms of percentage of callus production (40%) if compared to SP (11.3%) and clone GR that was not able to produce calli at all (Fig. 1S). Unfortunately, even if clone SP initially produced calli, they did not further proliferate in the following subcultures, so GR and SP ecotypes were not considered during the following steps of the experiment. On the contrary, clone HE produced abundant yellow and hard callus (Fig. 1A). Clone HE callus growth, monitored right after their transfer to EI medium, show a significant increase in callus dry weight that reaches

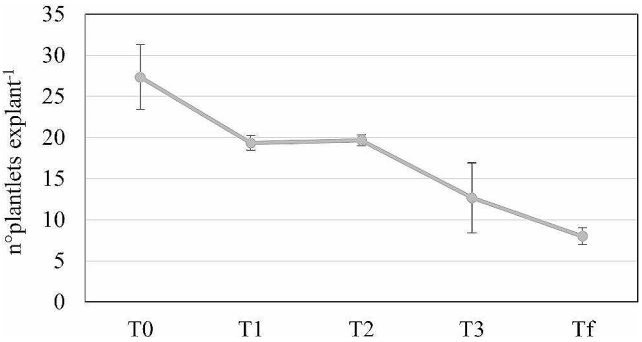

Fig. 2 Average number of plantlets per young flower bud in four subcultures from clone HE *I. pallida* micropropagated plantlets. Data are reported as mean values ± S.E

a plateau after 20 days in culture. Clone HE callus reaches a final dry weight of on average 35 g, showing a positive response to the culture medium (Fig. 2S). When clone HE callus is transferred on EI medium it starts to produce well developed white embryos (Fig. 1B). The embryos transferred on G medium are converted into new plantlets with a yield of 48%.

Plantlets obtained from embryos (Fig. 1C) were repeatedly subcultured on M medium, data on the average number of new shoots (Fig. 1D) produced per plantlet were taken in order to obtain the multiplication rate, fundamental parameter for the planning of an industrial scale up of *I. pallida in vitro* production (Fig. 2). The rooting of the new formed plantlets was spontaneous on the multiplication medium used (M), so it was not, necessary to use a specific rooting medium. In the Fig. 2, the multiplication rate is reported for four successive subcultures: a decrease in number of plantlets produced can be observed on clone HE, showing a progressive loss of multiplication potential.

Somatic embryogenesis on clone HE leaf tissue

To overcome the observed decrease in plantlets production, an alternative regeneration technique was applied starting from explants taken from leaves of in vitro clone HE plantlets. Results related to the two types of leaf explant used (5 and 3 mm of size) showed a significantly higher response

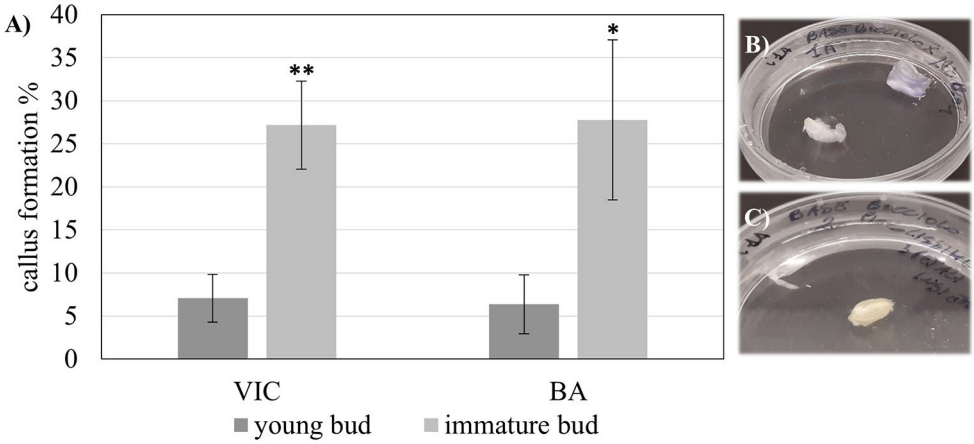

Fig. 3 Differences in percentage (%) of callus formation from young and immature bud explants of *I. pallida* BA and VIC ecotypes (**A**) and different explant types used for somatic embryogenesis of *I. pallida* BA and VIC ecotypes: portions taken from young flower buds (**B**) and whole immature flower buds (**C**). Data are reported as mean values ± S.E.; * indicates significant difference (t-student test, $p \leq 0.05$*; $p \leq 0.01$**)

Table 2 Percentage of callus formation, monthly callus growth index (GI) expressed as fresh (FW) and dry (DW) weight, n°of embryos (g_{dw} callus^{-1}; explant^{-1}) and n° of plantlets obtained in two subcultures of *I. pallida* clone HE, by somatic embryogenesis starting from leaf and young bud explants

HE	Callus formation (%)	GI (FW)	GI (DW)	*n*°embryos (g_{fw} callus^{-1})	*n*°plantlets explant^{-1}
Buds	40 ± 4 **ns**	0.4 ± 0.1	0.9 ± 0.1 **ns**	47 ± 12**	19.7 ± 0.7**
Leaves	43 ± 3 **ns**	0.6 ± 0.0**	0.9 ± 0.2 **ns**	13 ± 2.1	6.5 ± 1.4

Data are reported as mean values ± S.E.; * indicates significant difference (t-student test, $p \leq 0.05$*; $p \leq 0.01$**)

Table 3 Growth Index (GI), expressed as fresh (fw) and dry weight (dw) of calli, number of embryos and plantlets obtained from *I. pallida* young and immature buds belonging to BA and VIC ecotypes

Ecotype	Callus GI_{fw}		Callus GI_{dw}		*n*°emb g_{fw} callus^{-1}		*n*°emb explant^{-1}	
	Young bud	Immature bud	Young bud	Immature bud	Young bud	Immature bud	Young bud	Immature bud
VIC	0.4 ± 0.0 **ns**	0.4 ± 0.1 **ns**	1.2 ± 0.2*	0.6 ± 0.1	75.5 ± 15.8 **ns**	61 ± 13.9 **ns**	8.3 ± 0.9 **ns**	37.3 ± 13 **ns**
BA	0.4 ± 0.1 **ns**	1.2 ± 0.4 **ns**	0.7 ± 0.2 **ns**	0.3 ± 0.1 **ns**	47 ± 1	248 ± 57.9 *	13.3 ± 1.7 **ns**	16 ± 7.2 **ns**

Data are reported as mean values ± S.E.; * indicates significant differences between the explant types (t-student test, $p \leq 0.05$)

of 5 mm explants in terms of callus production (43%) if compared to the smallest ones (27,7%) (Fig. 3S); as a consequence, we decided to focus on callus, embryos and plantlets production of this type of explant.

Comparing the performances of flower bud and leaf tissues belonging to clone HE, no significant differences can be found both in terms of percentage of callus formed and of callus growth (Table 2). The analysis of the number of embryos and plantlets produced by young flower buds and leaves showed a significant difference among the explants, with a lower number of embryos and plantlets produced by leaf explants (Table 2).

Somatic embryogenesis on clone BA and clone VIC immature flower buds

The somatic embryogenesis technique was also performed on two other *I. pallida* ecotypes, BA and VIC, starting from two different types of explant, the first type consisting of portions of inner tepals taken from young buds (Fig. 3B), the second one composed by whole buds that still do not show a visual differentiation of outer and inner tepals (Fig. 3C).

The percentage of callus production was analogous for BA and VIC ecotypes and was significantly higher for immature flower bud explants, for both the ecotypes (Fig. 3A). Moreover, monitoring callus weight it was possible to see a higher production of dry weight in callus proliferated from young bud explants of clone VIC (Table 3). Considering the embryos production phase, no significant differences can be observed between young and immature buds except for the number of embryos of clone BA that results higher in immature flower bud explants (Table 3).

Quantitatively, no significant differences were found in the regeneration ability of the two types of explant used; however, as reported in Fig. 4 (A, B) the use of immature flower buds allows a more rapid regeneration procedure; in particular, the conversion into plantlets of embryos obtained from immature buds took place in a third of the time spent by those produced by young buds.

Plantlets obtained from the two types of explant were transferred to M medium and their multiplication was

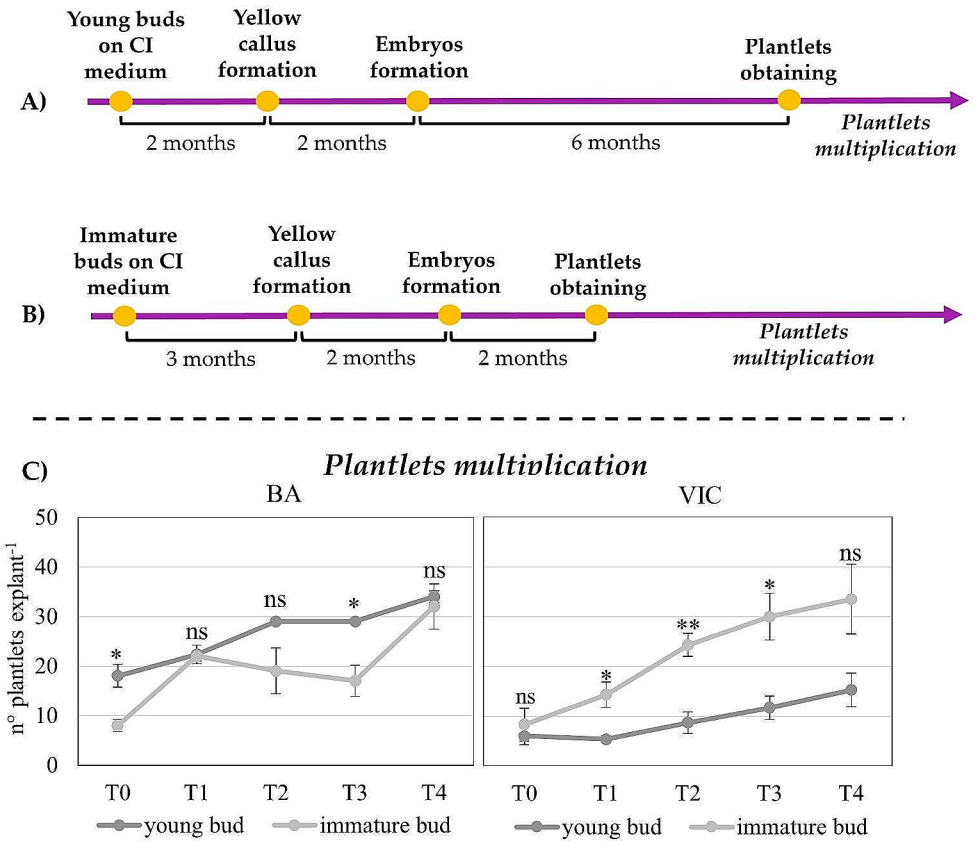

Fig. 4 Development timelines of the main steps of the somatic embryogenesis performed on young (**A**) and immature (**B**) flower bud explants and average number of sprouts per young and immature bud in four subcultures from clone BA and clone VIC *I. pallida* micropropagated plantlets (**C**). Data are reported as mean values ± S.E.; * indicates significant difference (t-student test, $p \leq 0.05$*; $p \leq 0.01$**)

monitored for both the ecotypes: the plant production rate increases in time in plants produced from both types of explant, even if with different behaviour, but after three months the same number of plantlets was obtained (Fig. 4C).

Acclimatization of clone BA and clone VIC micropropagated plantlets

BA and VIC plantlets obtained with the somatic embryogenesis technique with immature flower buds were subjected to an acclimatization protocol, in order to evaluate their ability to grow and adapt to field conditions. The acclimatization was performed introducing a step of pre-acclimatization in Magenta® vessels with ventilated caps. This procedure showed promising results, and at the end of the acclimatization, clone BA plants showed a survival percentage of 53% while clone VIC showed a higher percentage (70%).

Iridals phytochemical profile of clone VIC

The chromatographic profile of in vitro plantlets was examined in leaf explants and compared to that obtained from freshly harvested rhizomes of acclimated and mother plants. In vitro plantlets (Fig. 5A) showed a very similar chromatogram to those of acclimated and mother plants rhizomes (Fig. 5B), that have the same chromatographic profile (data not shown); this indicates a high uniformity in the metabolic profile. The chromatograms reported in Fig. 5 showed two large peaks at retention times between 27.5 and 29.5 min, respectively, along with minor peaks. The analyses carried out allowed to identify the two main peaks as iridals. The graph of Fig. 6 reports the concentrations of the two iridals in all the studied samples. Interestingly, in vitro plantlets show the higher concentration of both iridals. In all the three analysed tissues, the relationship between iripallidal and iriflorental was respected (60:40).

Discussion

The traditional propagation method used for *I. pallida* is a critical aspect of its production chain, as it requires a high amount of manpower, leads to the loss of a significant quantity of sealable material, and consequently results in substantial economic losses that are unsuitable for small and medium sized rural realities cultivating it (Belletti et al. 2013; Pezzarossa et al. 2020). However, numerous studies show that the somatic embryogenesis is a very effective technique that gives promising results for *Iris sp*. plants production. Jéhan et al. (1994) applied for the first time this technique on *I. pallida* starting from different organs (roots, leaves, apices and young flower buds) and the results showed

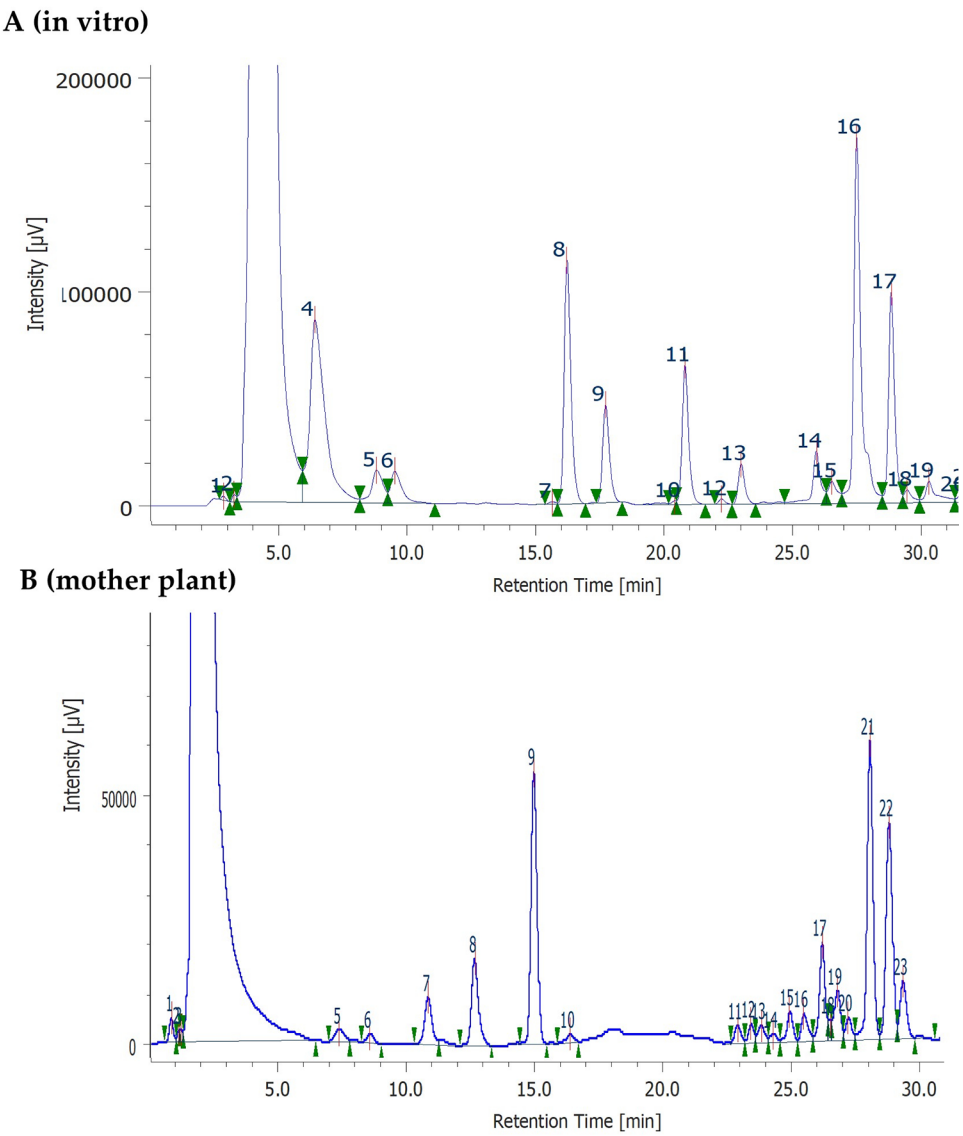

Fig. 5 Chromatograms of the extract from leaves of in vitro plantlets (**A**) and from rhizomes of *I. pallida* mother plants (**B**)

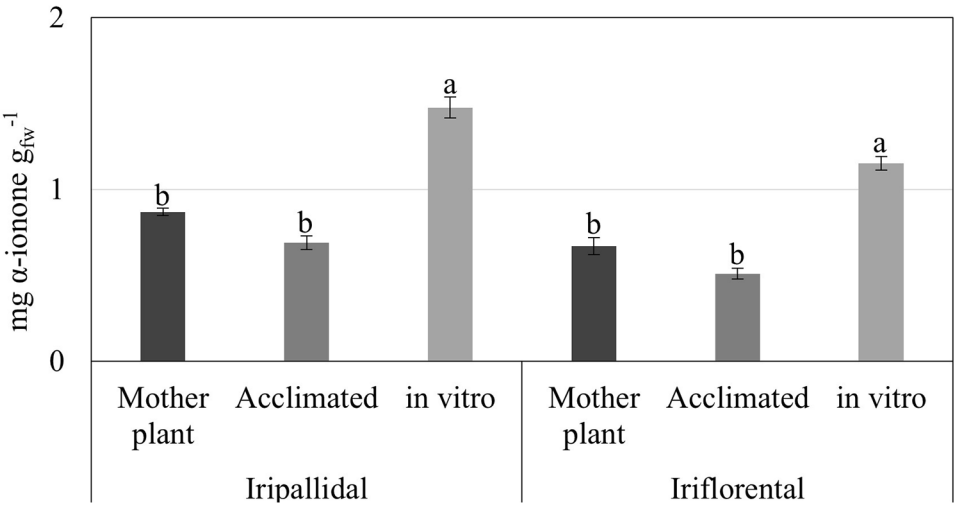

Fig. 6 Iridals (iripallidal and iriflorental) content (mg α-ionone g_{fw}^{-1}) in leaves of *I.pallida in vitro* plantlets and in rhizomes of acclimated and mother plants. Data, reported as mean values ± S.E., were subjected to analysis of variance (ANOVA) and different letters indicate significant differences among means (Tukey post-test, $p \leq 0.05$)

that young flower buds were the explants that provided the highest regeneration rates. More recently, Lucchesini et al. (2017) reported that axillary flower petals were the more performing explants for *I. pallida* micropropagation and that was possible in six months to obtain 150 plantlets starting from a single flower bud. In our experiment, an analogous number of plantlets was obtained in almost half of the time (four subcultures; 3,5 months). This result was due also to the use of a different sequence of media in comparison to previous protocols and to a different media composition (Lucchesini et al. 2017). During somatic embryogenesis from young buds performed on the three ecotypes of orris selected (GA, HE, SP), the ecotypes responded differently even if cultivated on the same medium, with clone HE showing the best performances during callus induction and proliferation, demonstrating that genotypes have different natural predisposition to respond to the somatic embryogenesis technique itself and to hormones that make up the medium (Hill and Schaller 2013) . However, clone HE showed a loss of regeneration potentiality during the multiplication phase. Previous studies demonstrated that *I. pallida* effectively responded to somatic embryogenesis performed on leaves (Lucchesini et al. 2017; Jéhan et al. 1994); thereafter, somatic embryogenesis on leaves was conducted on clone HE to incentivize plantlets production. Differently from what was previously done in the aforementioned studies, two types of leaf explants were tested in order to optimize the regeneration procedure. The results demonstrate the ability of this ecotype to produce callus, embryos and new plantlets starting from bigger leaf explants (5 mm), but not to perform an efficient subsequent plantlets multiplication. The somatic embryogenesis technique was also tested on other two *I. pallida* ecotypes, VIC and BA, using flower buds taken at different stages of development (young and immature). Even if there were no significant differences among the two types of explant in both ecotypes in terms of percentage of callus formed and plantlets produced, it was monitored a reduction of the time required for the conversion of embryos formed into new orris plantlets, going from six to two months (Fig. 4); this confirms that explants youth plays a fundamental role in somatic embryogenesis effectiveness (Kothari et al. 2010), related to its ability to rapidly reach the stage of totipotency necessary to produce embryos (Jevremović et al. 2006). Considering that this species avoids humidity excess, preferring dry conditions and loose soils (Pezzarossa et al. 2016; Ieri et al. 2022) we introduced a pre-acclimatization phase that helps plants to face the acclimatization stress, by reducing humidity inside the container and ameliorating the functionality of leaves apparatus. The use of perlite in the subsequent step allows to continue the avoiding of humidity and to permit the development of roots. The percentage of survival obtained with clone BA (53%) results slightly lower than the one obtained with VIC (70%).

The results related to *I. pallida* phytochemical profile are consistent with the literature, which reports two iridals (iripallidal and iriflorental) as the most abundant compounds in the fresh rhizomes of *I. pallida* (Bicchi et al. 1993). Iripallidal and iriflorental are isomers differing for the position of a double bond in the molecule and are the precursors of the irone compounds (cis-α-irone e cis-γ-irone, respectively) that form after two-three years in air dried rhizomes and are used by the perfume industry for their violet scent (Kukula-Koch et al. 2015; Mykhailenko 2018). In Lucchesini et al. (2017), a comparison between chemical profiles of rhizomes belonging to *I. pallida* mother and micropropagated plants both cultivated for three years was reported. In this study, the quantification of iridals and the phytochemical profile of in vitro plantlets was investigated for the first time, showing a clear correspondence of the chromatogram with acclimated and mother plant's one and, more in general, with *I. pallida* profile (Pezzarossa et al. 2020). Our results demonstrate that iridals were already present in tissues of young in vitro plantlets, further respecting the same iridals typical proportion (60:40). In vitro plantlets phytochemical profile could represent a marker that can be used to confirm their stability, that until now was performed using RAPD markers only on other Iris species (Cerasela et al. 2014).

In conclusion, the somatic embryogenesis technique performed with the procedure carried out in this study showed to be effective to propagate *I. pallida* plants; this study highlighted for the first time the diverse aptitude to somatic embryogenesis of different *I. pallida* ecotypes, both in terms of regeneration and acclimatization abilities. Moreover, the best procedure resulted the one performed starting from immature flower buds as explants, as it allows to significantly speed up the process of multiplication, fundamental aspect for industrial purposes. The acclimatization procedure, even if it gave worthy results thanks to the introduction of a pre-acclimatization phase, could be further improved. Further investigation could concern genetic stability with molecular techniques to confirm equality with the mother plant.

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Data availability The raw data supporting the conclusions of this article will be made available by the authors on request.

Declarations

Conflict of interest Author Giorgiana Chietera was employed by the company LMR Naturals by IFF (International Flavors & Fragrances SAS) and declares no conflict of interest. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships.

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